A Role for Regulatory T Helper Type 1 Cells in Interferon-β Therapy for Multiple Sclerosis

THESIS

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Nicholas Najib Jarjour

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Thesis Committee:

Professor Amy Lovett-Racke, Advisor, Professor Christopher A. Taylor, and Professor Yuhong Yang

Abstract

Multiple Sclerosis (MS) is a chronic disease of the Central Nervous System (CNS) characterized by inflammatory demyelination. The resulting effects on neuronal conduction lead to functional deficits affecting gait, vision, muscular function, and other basic processes, which occur in a relapsing/remitting or progressive manner. A number of the most effective treatments for MS are formulations of the human signaling molecule Interferon-β (IFN-β). However, these treatments' mechanism of action in the context of MS is not well established. We have previously identified a population of effector CD-4+ T cells that self-regulates via up-regulation of the immunosuppressive cytokine interleukin-10 (IL-10) in a mouse model of MS. We hypothesize that induction of IL-10+ T helper type 1 (Th1) cells is a key contributor to the efficacy of IFN-β therapy in MS. If this population is increased in MS patients receiving IFN-β treatment, this would strongly support a contribution to clinical benefit. If these cells are essential in IFN-β's mechanism of action, IL-10+ Th1 cells could be used as a marker of therapeutic efficacy and as a target for drug development.

We have developed a methodology to study effector CD-4+ T cells *ex vivo* to examine the expression patterns of the cytokines (signaling molecules) IL-10, Interleukin-17 (IL-17), and Interferon-γ (IFN-γ) by flow cytometry and enzyme linked immunosorbent assays (ELISAs) to establish the presence or absence of the target IL-10+

population and its characteristics. We have analyzed this population in 3 MS patients pre- and post-initiation of IFN-β therapy, as well as in an additional cohort of 2 MS patients pre- and post-initiation of the alternative MS treatment copaxone. Our data demonstrates no alteration in the IFN-γ+/IL-10+ double positive population after IFN-β or copaxone treatment.

Acknowledgments

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Vita

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Chapter 1: Introduction

Multiple Sclerosis (MS) is a devastating disease of the central nervous system (CNS) which has major effects on patients' lives. The most dramatic symptom which can occur in MS is paralysis, but gait, vision, cognitive function, and balance, among other processes, are commonly affected[1]. Perhaps surprisingly for a disease with such severe symptoms, it is predominantly recorded in modern history. While the first ostensible case of MS occurred in 1395 in Holland, the first concrete medical definition for the disease was developed by Charcot, in 1868[2]. There is no established cause for MS, but simply both anecdotal and published correlations which remain more or less controversial, among which are correlations with high latitudes and the developed world[1,3,4,5]. In the United States, there may be 350,000 MS cases, or more[3]. A similar number of cases have been reported in Europe[4]. Globally, over 2.1 million cases are estimated[5]. In Denmark, 154.5 cases per 100,000 people have been reported, or an incidence of over 1.5%[6]. MS may be increasing in incidence in the developed world, but this remains uncertain. Gender bias is a feature of multiple sclerosis, with three or more women suffering from the disease for every two men[1].There is no cure for the disease, current treatments simply limit disease progression, without reversing pathology in progressive forms of the disease. MS treatments possess major side effects.

A debate in the field is whether MS is one disease or many, as it presents in a number of different ways[1]. 55% of cases are relapsing/remitting (RRMS), which phenotype is characterized by attacks followed by nearly complete recovery or residual deficits[1]. RRMS patients typically advance to secondary progressive disease (SPMS, 30% of total cases), which exhibits slow, constant deterioration[1]. 10% of total MS cases are primary progressive (PPMS), with a similar course to SPMS, and 5 % benign (characterized by little to no deterioration), as well as rare acute cases (which progress more rapidly)[1]. Finally, clinically isolated syndromes, such as neuromyelitis optica, often present before the defined onset of MS, begging the question of how they relate to MS[1]. This complexity of clinical presentation leads to the question of whether different triggers lead to each form of MS; however, what initiates MS is still unknown.

MS is believed to be caused by an attack by the body's own immune system (termed an autoimmune response) against the myelin sheath of neurons, which is essential for proper neuronal conduction[1]. This results in impaired neuronal function, neuronal death, and "scarring" (gliosis, or recruitment of glial, supporting cells to form a scar-like site)[1]. The myelin sheath is destroyed in parts of the brain in MS, which can be visualized by a contrast agent called gadolinium. Areas of active demyelination appear as bright spots on MRI, while so-called "black holes" represent regions with axon (and myelin) loss[1]. It is believed that exacerbations (attacks in the relapsing/remitting form of MS) and new symptoms correlate with demyelination of the regions of the CNS corresponding to those functions affected. Active lesions exist during periods of silent

disease, leading to the idea that clinical disease occurs when the built-in functional reserve in the CNS is eroded. The essential problem of MS is that once a neuron in the CNS is lost, it will never be replaced.

Disease: As MS is an immune mediated disease, the constraints on the development of an immune response in the CNS are essential to a discussion of the disease. The master regulators and effectors of a specific (or adaptive) immune response are T cells, and T cell regulation is the crux of tolerance and autoimmunity. During development of T cells in the thymus, negative selection takes place to eliminate or anergize (render unreactive) autoreactive T cells, or to divert them into an immunosuppressive regulatory T cell lineage (Treg). Despite the presence of any "escaped" potentially autoreactive T cells, further barriers remain to an immune response in the CNS. Regions of the body like the CNS are considered to be "immunoprivileged," meaning that immune responses are prevented or heavily restricted within the site. One major element of CNS privilege is the Blood Brain Barrier (BBB), an impressively restrictive barrier to cells and molecules passing into the CNS. However, over the last few decades, it has become clear that this immunoprivilege is more tenuous than previously thought and that the BBB is not completely impermeable to immune cells[7]. For MS to develop, these hurdles must be surmounted by autoreactive T cells.

Two key steps in MS development are access to the brain for autoreactive T cells and the development and maintenance of an autoreactive response, as even healthy

individuals appear to have myelin specific, potentially autoreactive T cells[8]. With regard to the first, as stated, the BBB is a highly stringent regulator of substances and cells entering the CNS. Though there are now publications demonstrating that effector T cells may be able to conduct CNS surveillance under homeostatic conditions, naïve (or antigen-"target"-inexperienced) T cells do not gain access to the CNS[7]. While alteration of the BBB's permeability to T cells does appear to occur in MS, the key step in MS development may be the generation of memory T cells which are reactive with myelin, as might occur when cross reactive virus specific effector T cells are activated in infection[9]. Such cells could pass the BBB to start an immune response in the CNS. Regarding the second key requirement, immune responses are not intended to continue indefinitely and immune cells have a finite lifespan, while Tregs begin to develop in an environment with excessive tissue damage, late in the immune response[10]. Thus, MS does not only require an autoimmune response, but also dysregulation of the controls that would terminate the response, at least in the case of progressive disease. The existence of relapsing disease likely implies that there is control of the disease at points, but something breaks that tenuous tolerance. Such hints provide hope for immunotherapies to restore tolerance in MS.

Epidemiology: While MS' etiology remains unclear, a number of studies and anecdotes provide possible clues to its etiology. The Faroe Islands, situated halfway between Norway and Iceland, were subjected to a major influx of soldiers during World War II, and MS appeared soon after among the natives[1]. This would support an infectious

agent as the trigger for MS as this short time frame is likely insufficient for changes in hygiene, diet, and similar factors to take effect. A viral infection could potentially break tolerance for myelin by inappropriately over-activating the immune system or activating cross-reactive T cells coincidentally specific for both a viral antigen and a myelin antigen. Since the 1960's and perhaps earlier, viruses have been considered prime candidates in the genesis of MS[11]. Studies have shown measles virus specific antibodies in the cerebrospinal fluid and serum, circulating Epstein Barr virus specific CD-8 T cells, and hepatitis G virus DNA integrated into cells in the CNS in MS patients[11,12,13]. However, correlating infectious agents with MS is often confounded by better reporting and diagnosis after development of a modern infrastructure, as is true for other products of development like advanced hygiene (which could lead to altered development of the immune system because of less microbial stimulation). Additionally, a single virus has not been reproducibly found in the human CNS.

An alternative hypothesis recently advanced is that an unnatural amino acid (azeditine-2-carboxylic acid) in sugar beets resembling proline causes altered self-peptides which are recognized by the immune system or promote altered stability of myelin, leading to autoimmunity[14]. In the case of the altered amino acid model for MS, if this novel amino acid were incorporated into antigens for T cells, no regulatory mechanisms would have deleted T cells specific for these antigens as they had not previously been seen by the body. Regarding altered stability of myelin, if myelin breakdown occurred at the wrong time, or if myelin breakdown products arrived at the

wrong place (in immunological terms, such as a site of infection), an immune response could potentially be mounted against a self-protein. Correlative data supporting this is that Charcot first described MS one generation after Napoleon ordered the cultivation of sugar beets to replace imported sugar, due to a blockade[14]. Also, an outbreak of "swayback," a neurological disease in sheep, occurred in lambs in Alberta after their mothers were given a beet silage diet[14]. This correlative data forms an interesting hypothesis which will have to be confirmed by future studies.

Other groups have attempted to assign a mechanism to the known correlation between high latitude and MS, namely that lower vitamin D levels in areas with less sunlight drive MS development[15,16]. A study of Iranians living in Tehran (Figure IA) in 1989 and 2008 showed that incidence increased dramatically over two decades, and a 3.11 female to male case difference was observed in 2008, an increase over that in 1989[17]. This relates to vitamin D at two levels: first, urbanization of Iran is increasing and Tehran has experienced dramatic population growth, possibly linking a relatively sun-deprived urban setting with MS, and second, women were required to wear fully enveloping dress after the 1979 Islamic Revolution[17]. Increasing urbanization and decreased exposure to sunlight are trends across much of the world, and thus could drive further cases of this devastating disease. A better understanding of this subject is critical.

With regard to one final publication on the etiology of MS, a PLoS One study of Danish cell phone users was conducted to establish the effect of cell phone usage on rates

of MS incidence after a study controversially showed that a 900 MHz field from a phone caused BBB breakdown in rats[6]. This study established that cell phone usage does not appear to correlate with MS, with the possible exception of female users[6]. However, this population was underpowered in the study[6]. This area of research illustrates how many possibilities have been considered as causative agents for MS.

In addition to environmental factors, there is clearly a genetic component as shown by moderate correlation in disease status between identical twins, though genetics do not solely determine disease[18]. From this and the previous examples, it becomes clear that the cause of MS is likely multifaceted and a rigorous understanding is currently beyond our knowledge.

Treatment: Because the etiology of MS remains unclear and targeted immunotherapies remain distant, it is imperative that a better understanding of current treatments for MS be achieved to use them more effectively and to refine treatments. MS treatments, while not curative, can delay disease progression and delay attacks, maintaining function. While new MS treatments are being developed and approved by the FDA, the current treatments fall into the following general categories: competitive inhibitors of self-peptide binding to major histocompatibility complex proteins (MHC, recognized with peptide by T cells) (Copaxone?), interferon-β (IFN-β) formulations (Avonex, Rebif, Extavia, and Betaseron), trafficking inhibitors (Gilenya and Tysabri), chemotherapeutics (Novantrone), and inhibitors of immune cell function (Copaxone,

Tecfidera, and IFN-β)[19]. However, we do not understand many of these drugs' mechanism of action completely, including those based on IFN-β.

The IFN-β drugs can reduce exacerbations, increase time to new exacerbations, slow progression of disease, and decrease the number of active lesions[19]. However, the mechanism for such efficacy is uncertain. Type I interferons, like IFN-β, are produced by the body primarily in the context of viral infection. In this setting, the proinflammatory and antiviral features of these cytokines typically predominate, preparing uninfected cells to defend against viral infection, as well as recruiting and supporting immune cells. However, IFN-β also possesses numerous immunomodulatory functions, such as inhibiting antigen presentation to T cells, blocking immune cell proliferation (such as T cells), inducing immunosuppressive cytokines, inhibiting Antigen Presenting Cell (APC) costimulatory properties, and impairing trafficking of the cells of the immune system (Fig. I)[20,21]. These functions likely restrain the interferon response, especially towards the end of an infection. In the context of a chronic immune attack, like MS, IFN-β could promote the cessation or limitation of the autoimmune response. Our studies focus on a role for IFN-β in the induction of interleukin-10 (IL-10) in effector T cells, which is a potential mechanism of self-regulation by autoreactive mediators. In brief, IL-10 can block differentiation of T cells, impair proinflammatory cytokine signaling, prevent activation of macrophages, block effector function, reduce costimulation, and limit antigen presentation (Fig. II)[22]. IFN-β has been shown to induce IL-10 (in dendritic cells)

in a manner dependent on cyclic AMP response element binding protein (CREB) and inhibition of glycogen synthase kinase 3 (GSK-3) (Fig. III) [23].

A previous study has established that IFN-β increases IL-10 protein and mRNA levels in both healthy individual and MS patient peripheral blood mononuclear cells (PBMCs, essentially lymphocytes and monocytes) *in vitro*[24]. The same publication demonstrated that intramuscular injection of IFN-β induced higher serum IL-10[24]. Other studies have shown that Avonex increases IL-10 mRNA in MS patients' PBMCs, that Avonex also increases IL-10 levels in the cerebrospinal fluid (CSF), and that CSF IL-10 levels are correlated with clinical of efficacy of Avonex[25,26]. In contrast, Waubant *et al*. showed that serum IL-10 decreases with IFN-β treatment, but that IL-10 was typically elevated during the time period when lesions resolved[27]. Thus, there is strong evidence that IL-10 plays a role in IFN-β efficacy, but the question is, what population of cells is secreting the cytokine?

T cell populations: the markers CD-4 and CD-8 divide the two populations of human T cells. CD-4+ T cells are the key regulators of the immune response, complementing the (primarily) direct cytotoxic effects of CD-8+ T cells. CD-4+ T cells are subdivided into an ever-increasing number of subsets; this brief survey will focus on the four major types, T helper type 1, 2, and 17 cells (Th1, Th2, and Th17) and Tregs. The characteristic Th1 cell cytokine is IFN-γ, and Th1 cells are considered to be relatively inflammatory. They are essential in the optimum activation of macrophages

(the tissue resident immune cell responsible for killing pathogens and cleaning debris from the tissue) and are (along with Th17 cells) considered to be important in Experimental Autoimmune Encephalomyelitis (EAE) model of MS[28]. Th2 cells are less inflammatory and mediate a B cell based antibody-response; a stereotypical cytokine for Th2 cells is IL-4. Th17 cells make IL-17 (hence the name) and are critical in neutrophil recruitment, which are cells similar to macrophages in that they phagocytose pathogens, but differ in that they are short lived. While one form of EAE is Th17 cell driven, neutrophil recruitment is not observed in MS[28]. Tregs are generally defined by FoxP3 expression, the master transcription factor of the subtype, and are responsible for maintaining tolerance as well as limiting the immune response. Tregs can inhibit activation of T cells in contact and non-contact dependent manners. Tregs are considered to have a beneficial impact on MS disease (i.e. reduce disease). While these lines are elegantly demarcated, the experimental reality blurs lines between cell types.

One manifestation of this is the IL-10+ effector T cell population which has gained increasing prominence over the past decade and a half, with publications demonstrating that interleukin-4+, interferon-γ+, and interleukin-17+ T cells (T helper type 1, 2, and 17) all can make IL-10[29, 30,31]. This contrasts with traditional immunology in that inflammatory Th1 and Th17 cells "should not" make an immunosuppressive cytokine like IL-10. In fact, it was long believed that onlyTh2 cells made IL-10 (before the discovery of Tregs). Del Prete *et al*. critically rejected the dogma that IL-10 was a Th2 cytokine and established that both Th1 and Th2 type cells can secrete it[29].

Additionally, the authors showed that an IL-10+ Th1 cell shows reduced proliferation in response to antigen due to IL-10 mediated effects on APCs; IFN-γ production in response to antigen was also reduced[29]. While this population could be construed as solely Tregs, future publications elucidated that a subset of the population is distinct from the CD25+, FoxP3+ conventional regulatory T cell population and is in fact composed of effector T cells; their existence has been demonstrated in both humans and mice, though with the caveat that some publications have not definitively eliminated the possibility that the cells are IFN-γ+/IL-10+/FoxP3+ Treg cells (Fig. IV)[30,32,33,34,35,36]. Axtell *et al*. demonstrated that IFN-γ, IL-10, and IFN-β are intimately linked in the EAE model of MS by demonstrating that in a model of Th1 driven EAE, IFN-β efficacy required IFN-γ signaling and correlated with IL-10 levels[28]. IFN-γ/IL-10+ Th1 cells have been shown to provoke less severe disease in the EAE model, when transferred to induce EAE[34]. Similarly, TGF-β/IL-6 induced IL-17+/IL-10+ Th17 cells reduced EAE severity[30]. With regard to literature on other contexts in which these populations are important, Jankovic *et al*. demonstrated that IFN-γ+/IL-10+ effector T cells are critical in suppressing *Toxoplasma* survival and negatively regulate IL-12 production by APCs[35]. Another group demonstrated that IL-10+ effector T cells are contributors to the chronic infection state seen in leishmaniasis, a more negative role[36]. Patients infected with tuberculosis also exhibit IFN-γ+/IL-10+ Th1 cells, which likely contribute to the chronic infection state[32]. IL-27, TGF-β, IL-12, and TGF-β plus interleukin-6 all can induce IL-10 in helper T cells when provided during activation[31,34,37,38].A common thread in many of these publications is that repeated or further activation of effector T cells can induce IL-10, suggesting that

this is a self-regulatory mechanism[34,35,37]. As a corollary, these cells can be beneficial or harmful in the state of infection, either by reducing harmful inflammation or allowing the maintenance of a pathogen. In an autoimmune disease like MS, the former is naturally the relevant case.

IFN-γ+/IL-10+ Th1 cells are a promising area of MS research because of their demonstrated importance in the EAE model, the role of IL-10 in IFN-β efficacy in that model, and the immunoregulatory properties of IL-10[22,23,34]. Thus, we hypothesize that IFN-β induces IL-10+ Th1 cells in MS patients and that these cells self-and allo-regulate to reduce the immune response.

We have addressed this through the use of MS patient PBMC samples pre- and post- treatment with IFN-β, which we characterized by flow cytometry and Enzyme Linked Immunosorbent Assays (ELISAs) to search for alterations in our population of interest. If a role for IFN-γ+/IL-10+ Th1 cells is established, this could also be a means to determine how well a patient is responding to treatment at a more nuanced level than whether new lesions have developed. These cells could also be studied in the contexts of other treatments as a potential biomarker. Additionally, through a better understanding of this Th1 cell population, we would potentially contribute to our current understanding of MS development and how regulation is disrupted in the disease state.

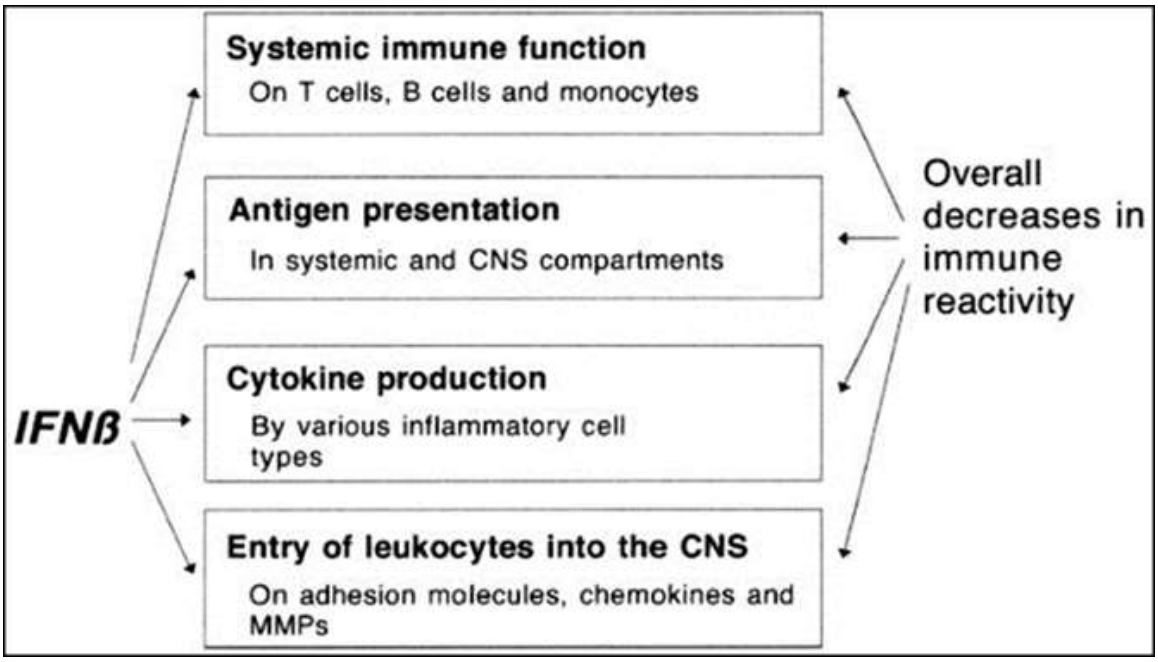

Figure I: Immunomodulatory roles for IFN-β. IFN- β is known to limit proliferation of immune cells, to down-regulate antigen presentation, to limit costimulation, to induce cytokines like IL-10 with immunoregulatory function, and to down-regulate adhesion molecules and matrix metalloproteases, which are required for entry by T cells into tissues[19]. Figure from Yong, V *et al*[19].

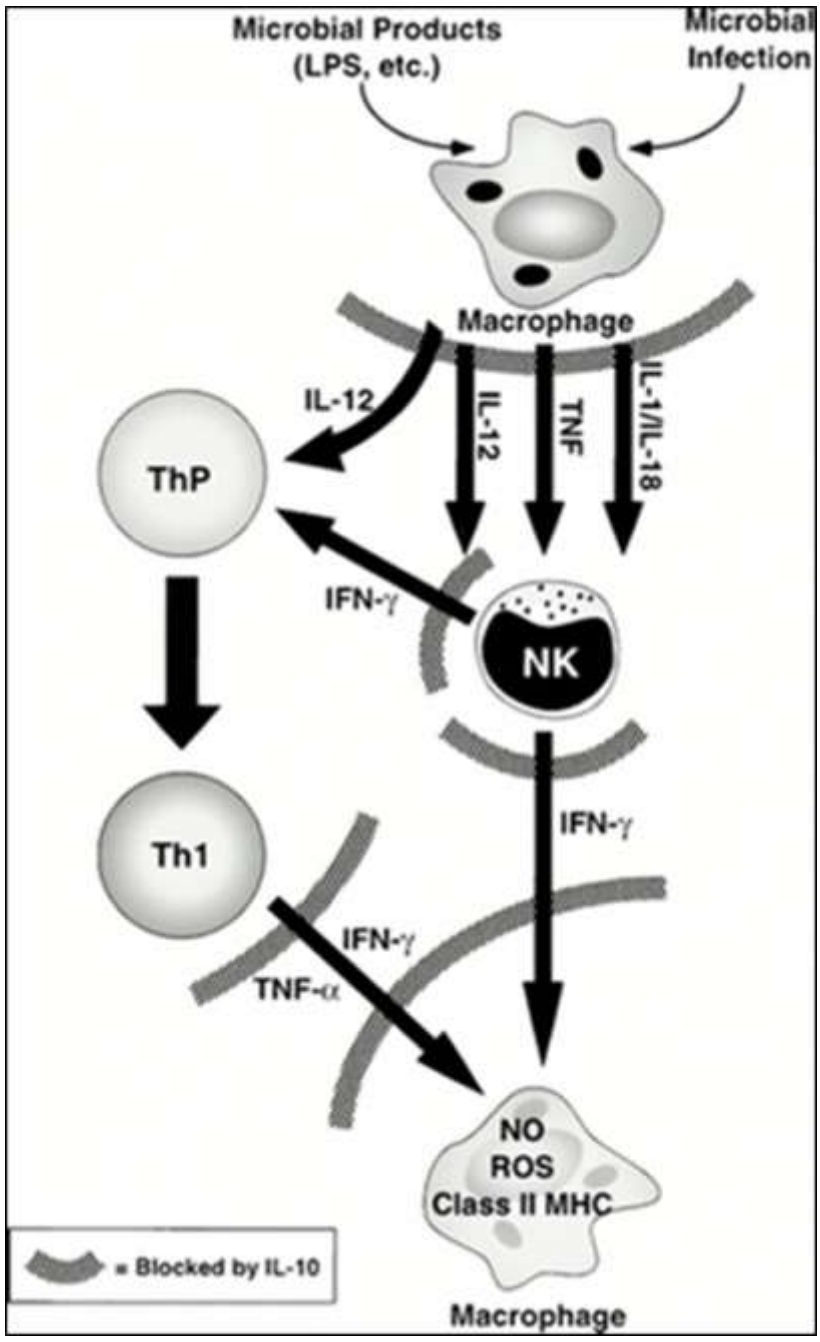

Figure II: Immunomodulation by IL-10. IL-10 possesses numerous means to suppress the immune response. Fig. III depicts some of these functions with solid gray bars indicating suppression of signaling by IL-10. The cytokine can block macrophage to T cell and macrophage to NK crosstalk, impair macrophage activation, and disrupt differentiation of T cells[22]. Figure from Moore, K *et al*[22].

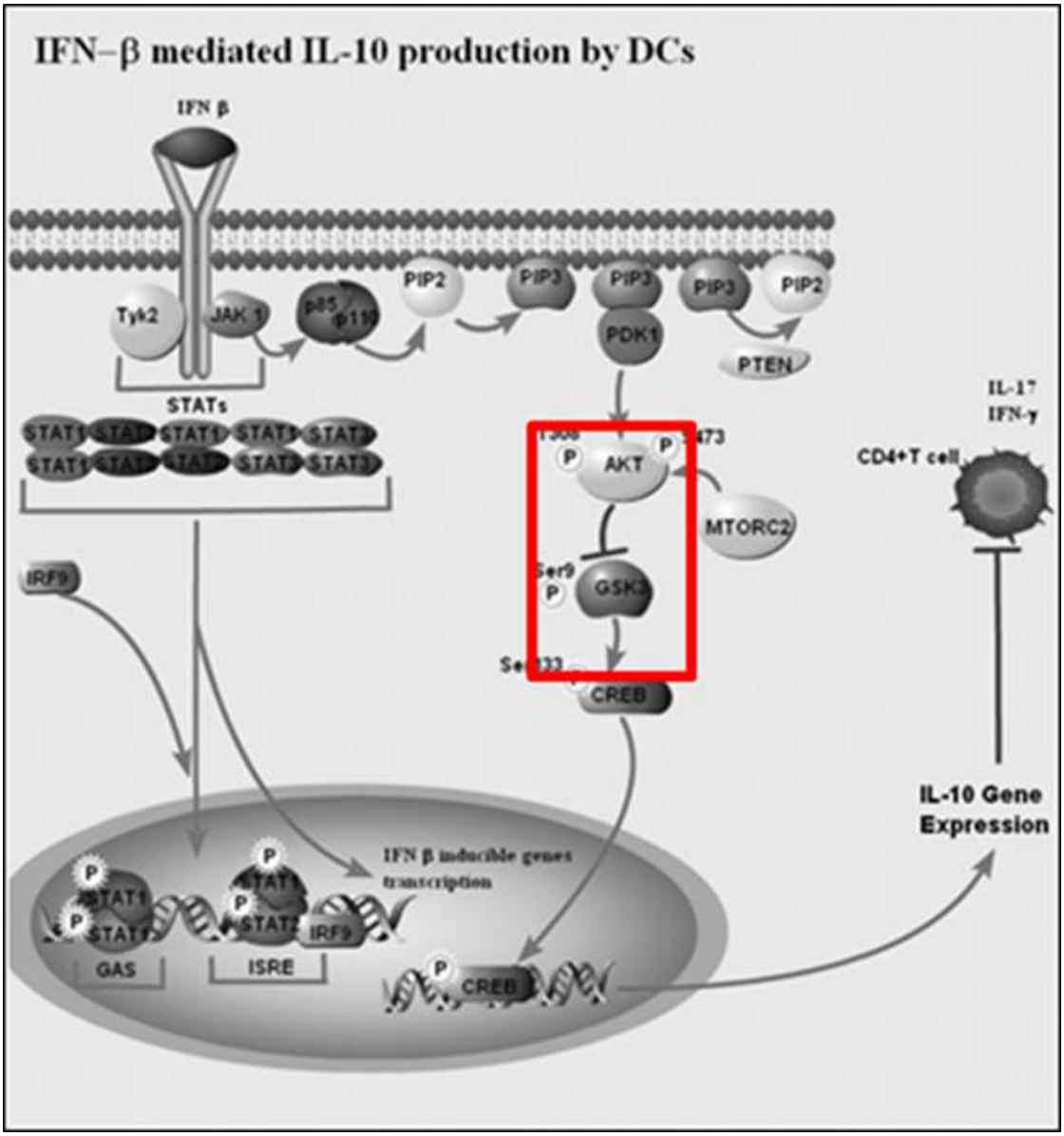

Figure III: A potential mechanism for the induction of IL-10 by IFN-β. Inhibition of GSK-3 and CREB activity have been shown to be required for IFN-β induction of IL-10 in dendritic cells[23]. Does the same hold true in T cells? Figure adapted from Wang, H *et al*[23].

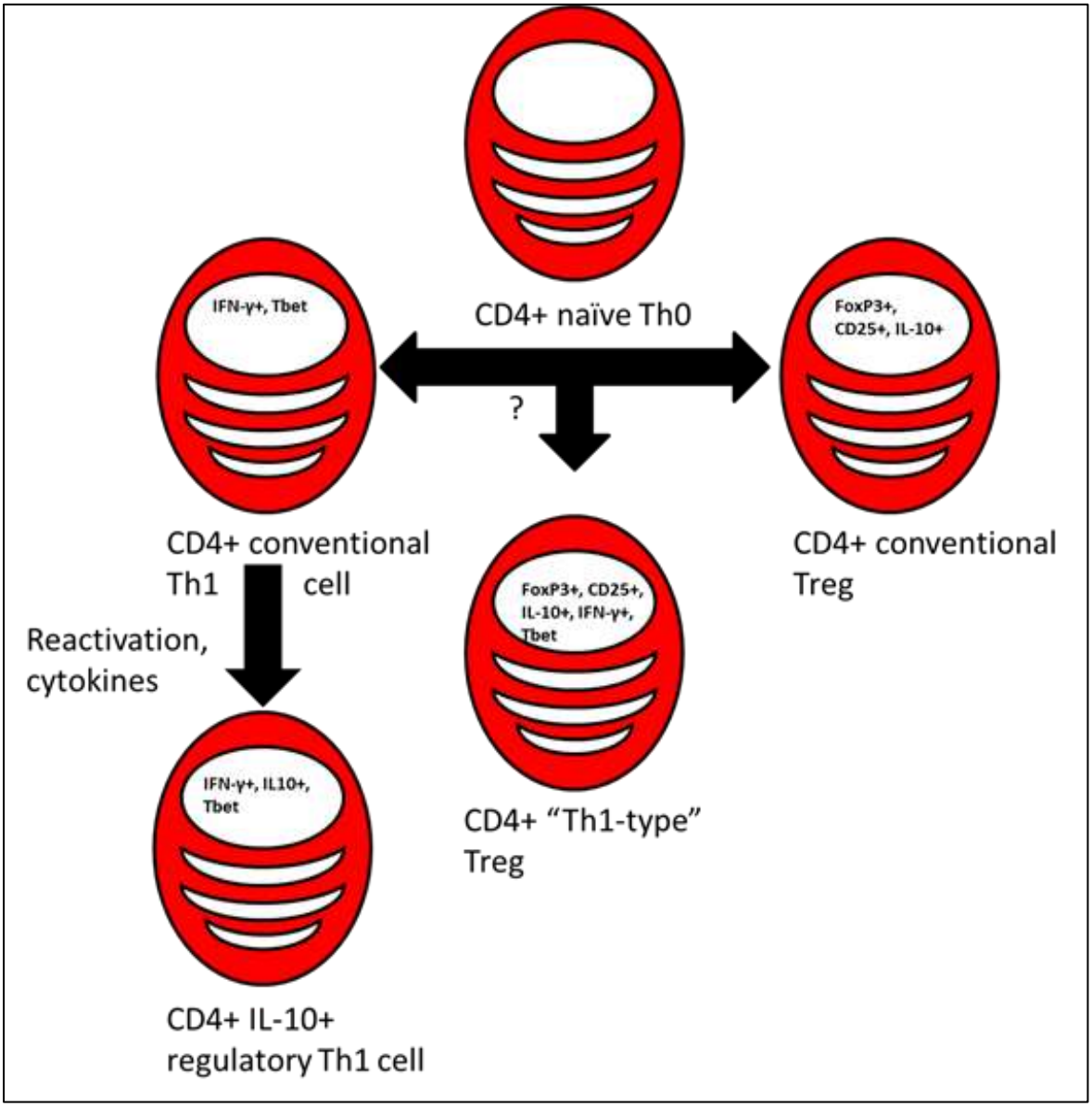

Figure IV: Th1 and Treg populations. A naïve T cell (Th0) can differentiate into multiple cell types, including those herein discussed. Conventional Th1 cells are IFN-γ+, Tbet+ (the characteristic Th1 transcription factor). Conventional Tregs are FoxP3+, CD25+ , often IL-10+. The focus of this study are CD-4+, FoxP3-, CD25-, IL-10+, IFN-γ+, Tbet+ effector Th1 cells, which appear to be derived from conventional Th1 cells in a manner promoted by reactivation and certain cytokines. "Th1 type" regulatory T cells are IFN-γ+,Tbet+, FoxP3+,CD25+, often IL-10+. This population has uncertain origins.

Chapter 2: Methodology

Peripheral blood mononuclear cell (PBMC) culture: MS patient PBMC samples were available from pre-treatment to up to 4 years of post-treatment depending on the patient. PBMCs encompass primarily lymphocytes (T cells, B cells, and Natural Killer cells) and monocytes (which give rise to macrophages). All samples were collected at the University of Texas Southwestern and stored in liquid nitrogen. Copaxone treated patient samples were obtained from a double blind study, thus copaxone stimulation was used to assay proliferation to determine which patients received placebo versus copaxone. Patient cohorts: 3 relapsing/remitting MS patients treated with IFN-β (Avonex or Betaseron) and 2 primary progressive MS patients treated with Copaxone. A mixed population of T lymphocytes and other cells was obtained by thawing the cells and resting the total population from 2-4 hours to remove adherent cells and to allow dead cells to clump for removal. Cells were cultured in human media (500 milliliters (mL) RPMI 1640, 5 mL 1 molar Hepes, 5 mL 10,000 International Units/mL Penicillin/10,000 micrograms/mL Streptomycin, 5 mL 200 millimolar L-Glutamine, and 25 mL heat inactivated Human Serum) with or without anti-CD-3 antibody stimulation. Anti-CD-3 antibody directly activates the T cell receptor complex without specific engagement of the receptor. While memory T cells, which have been previously activated, do not require additional stimulation, naïve, antigen-inexperienced T cells (Th0) require costimulation,

which can be provided with anti-CD-28 antibody. As we wished to study only the memory T cell population, we only provided anti-CD-3 stimulation.

Flow cytometry: After ~44 hours, PMA/Ionomycin was added to the anti-CD-3 treated condition to further enhance cytokine production (PMA is an activator of protein kinase C, while ionomycin is a calcium channel, both of which activate T cells). After one hour, GolgiStop (contains monesin) was added to block export of proteins from the Golgi, which aided detection of intracellular cytokines. Four hours later, supernatants and cells were collected separately for analysis by ELISA and flow cytometry. Flow cytometry consists of staining cells with fluorophore-tagged antibodies specific for different markers. The cells are then run through excitatory lasers one at a time to analyze levels of each marker on an individual cell through exciting the fluorophores. Flow cytometry staining groups consist of compensation controls, isotype controls, and test samples. Our compensation controls used unstimulated cells cultured in parallel with activated populations and were either unstained or stained with a single antibody. Unstained cells allow calibration of the voltages for the excitatory lasers used in flow cytometry so that positive population is within the range of detection. Single antibody stained controls allow compensation for overlap between different fluorophores' emission spectra. An isotype control consists of a non-specific antibody of the same isotype as the antibody for the marker of interest, which yields a value for background fluorescence. The markers of interest were: CD-8, CD-3, IL-10, IL-17, and IFN-γ. CD-3 and CD-8 are markers of T cells and cytotoxic T cells, respectively. By gating on the CD-

3+ (T cell), CD-8- (CD-4+ T cell) population, we were able to study CD-4+ T cells, our population of interest (Fig. V). Direct gating on CD-4 was not possible because the strong activation seen with our conditions resulted in down-regulation of CD-4. Intracellular staining was made possible through the use of a fixation and permeabilization kit. Before each antibody staining step, cells were treated with Fc block, a reagent which binds to the receptors for antibodies on the cells. Without this, antibodies would be non-specifically retained by cells. All samples were run on a BD FACSCanto II and all analysis was conducted with FlowJo. A note on gating, whenever the compact double negative population on IFN-γ+/IL-10+ or IL-17+/IL-10+ plots extended beyond the gate set by the isotype, gates were conservatively extended to encompass the entire population. 50,000 events were collected if possible, each event representing one cell. A flow scatterplot with respect to markers A and B has four quadrants, A-B-, A+B-, A+B+, and A-B+, clockwise from the bottom left. Each point on a scatterplot represents an individual cell. If a levels of a given marker on a cell fall above the background level determined by the isotype control, that cell is considered to be positive.

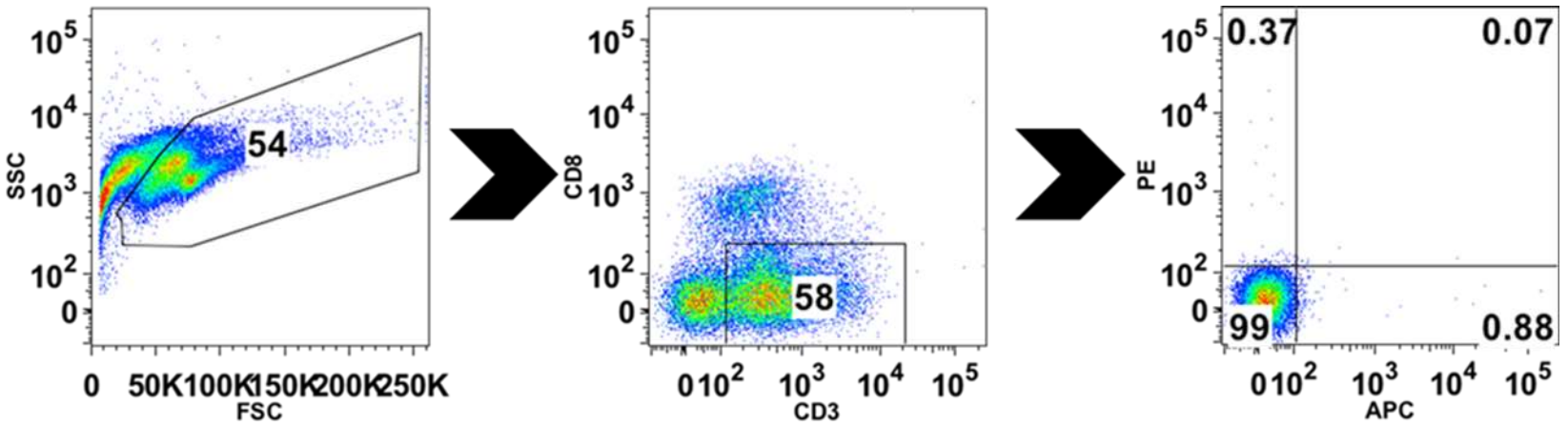

Figure V: Gating strategy. Forward Scatter (FSC, Size) and Side Scatter (SSC, Granularity) were used to gate on living cell populations (high SSC, low FSC are dead cells). Using the population selected by the FSC vs. SSC gate, CD-3 vs. CD-8 gating was used to select CD-3+,CD-8- cells, which are CD-4+ T cells. Isotype controls were used to set the threshold for a positive signal above background fluorescence in the population yielded by CD-3 vs. CD-8 gating. Thus, any cell in the test sample appearing as positive for a cytokine via this gating methodology will be alive, CD-4+, and exhibit greater than background staining for the cytokine. As noted in the text, gates were increased if the double negative population for IL-10 vs. IFN-γ were above the threshold set in accordance with the isotype. For rightmost isotype scatterplot, X axis APC fluorophore, Y axis PE fluorophore.

Enzyme linked immunosorbent assays (ELISAs): A technique using antibodies to detect levels of a specific protein with high accuracy and precision. To assay protein secretion levels, supernatants were collected from all samples, initially with slight PBS dilution due to washing the cells out of the wells and subsequently by withdrawing supernatants from wells without disturbing the cells. Protein levels were analyzed in our study by sandwich ELISAs, which involved coating a well with an antibody specific for the protein of interest, adding a sample which may or may not have contained that sample, and treating with another antibody specific for a different epitope (or binding site) on that protein. The second antibody was conjugated to biotin, which subsequently was exposed to a peroxidase-avidin conjugate (avidin and biotin bind in a high affinity manner). Addition of substrates ABTS and hydrogen peroxide led to a peroxidase-mediated electron transfer reaction from ABTS to H_2O_2 resulting in an ABTS product with a bright green color, which was quantified. Through the use of a dilution series of the protein of interest to construct a standard curve of concentration to absorbance, each of the experimental samples was plotted onto the standard curve to determine its concentration. ELISAs were conducted with duplicate samples.

^{3}H Thymidine proliferation assays: Cells were activated with α-CD-3 antibody alone and, after 48 hours, radioactive tritiated thymidine was added. As one of the four DNA nucleotides, thymidine, radioactive or not, is incorporated into DNA by proliferating cells. Thus, through provision of ^{3}H thymidine, total proliferation can be assessed by reading the total amount of radioactive thymidine taken up by cells. The cells

were harvested 18-24 hours later and radioactivity was quantified with a PerkinElmer TopCount NXT to determine proliferation. This assay was used to both confirm T cell activation while our methodology was being developed and to determine whether PBMC samples from the Copaxone study were from patients treated with placebo or copaxone.

Chapter 3: Results

Our first result was the identification of anti-CD-3 antibody treatment plus PMA/Ionomycin stimulation as optimal for inducing IL-10, IL-17, and IFN-γ production. As human cells exhibit extensive variation and are extremely sensitive to conditions, optimizing their treatment for maximal cytokine expression was essential to our studies. By comparing anti-CD-3 antibody, PMA/Ionomycin, and anti-CD-3 antibody+PMA/Ionomycin conditions, we were able to identify the last as conducive to maximal cytokine expression. This is logical as the most powerful activating conditions are likely to induce higher cytokine levels.

The second step was selecting appropriate antibodies and optimizing flow cytometry protocols. Flow cytometry antibody selection depends both on using an available fluorophore and using an antibody clone that specifically and accurately quantitates levels of the marker of interest. We conducted separate staining for IL-17 and IFN-γ in parallel to allow use of the PE fluorophore for both, as it provides the best staining. For IL-10, APC was used. For CD-8, FITC, and for CD-3, PerCP. Future experiments in progress use AlexaFluor405 and preceeding ones used eFluor450, illustrating how quickly the 8 channels for colors on a flow cytometer can become occupied. Additionally, the intracellular staining protocol for flow cytometry makes

staining a long and complex procedure. Considerable time and resources were devoted to troubleshooting this technique.

To address whether IFN-γ+/IL-10+ double positive Th1 cells are induced by IFN-β treatment, we reactivated memory T cells from MS patients treated with Avonex or Betaseron as described and profiled the total population by flow cytometry with respect to IL-10, IL-17, and IFN-γ to look for changes before and after patient treatment. Flow cytometry is quantitative as well as qualitative (at the single cell level) as fluorescence intensity increases with bound antibody, which increases with surface marker levels. Quadrant gates allow identification of double positive versus single positive populations, which is essential to allow us to identify the IFN-γ/IL-10+ double positive population of interest. As presented in Fig. V, our gating methodology selected living CD-4+ T cells. We did observe all three cytokines of interest, with the highest levels of IL-17and lowest of IL-10, as well as the largest single cytokine positive population being IFN-γ+ and lowest IL-17+ (Fig. VI). As seen in the IFN-β treated cohort flow cytometry studies, the IFN-γ+ population decreases in 2/3 patients, while the population stays roughly constant in 1/3. The IL-10+ population decreases in 1/3 and remains approximately constant in 2/3. The IL-17+ population increases in 1/3, decreases in 1/3, and remains roughly constant in 1/3. The IFN-γ+/IL-10+ double positive population decreases in 1/3 and remain approximately constant in 2/3. The IL-17+/IL-10+ double positive population is at ~0% in all patients at all times.

With regard to the second cohort, examining levels of IFN-γ+/IL-10+ double positive cells in a copaxone treated population both allowed us to observe whether any trend observed was IFN-β specific or not and to potentially contribute to a better understanding of copaxone efficacy. An identical methodology was used for this cohort as for the other. Maximal levels of cytokines were similar to those observed in the IFN-β cohort, as were the population percentages. Within the copaxone treated cohort (Fig. VII), the IFN-γ+ population increased in 1/2, while the population stayed roughly constant the other patient. The IL-10+ population appears to have remained constant in 1/2 and decreased in 1/2. The IL-17+ population increased in 1/2 and remained constant in the other patient. The IFN-γ+/IL-10+ double positive population is practically undetectable and remained roughly constant. The IL-17+/IL-10+ double positive population is at ~0% in all patients at all times.

While flow cytometry does quantitate protein levels of cytokines, the cytokines observed are retained intracellularly. Thus, examining extracellular cytokine levels in supernatants by ELISAs was essential. With regard to the ELISA data for the IFN-β cohort (Fig. VIII), all cytokines appeared to be expressed, though IL-10 was at an extremely low level. However, no significant differences were detected between patients or at different times. As for the previous cohort, in the copaxone cohort, all cytokines appeared to be expressed by ELISA (Fig. VIII), though IL-10 was again at an extremely low level. However, no significant differences were detected between patients or at different times.

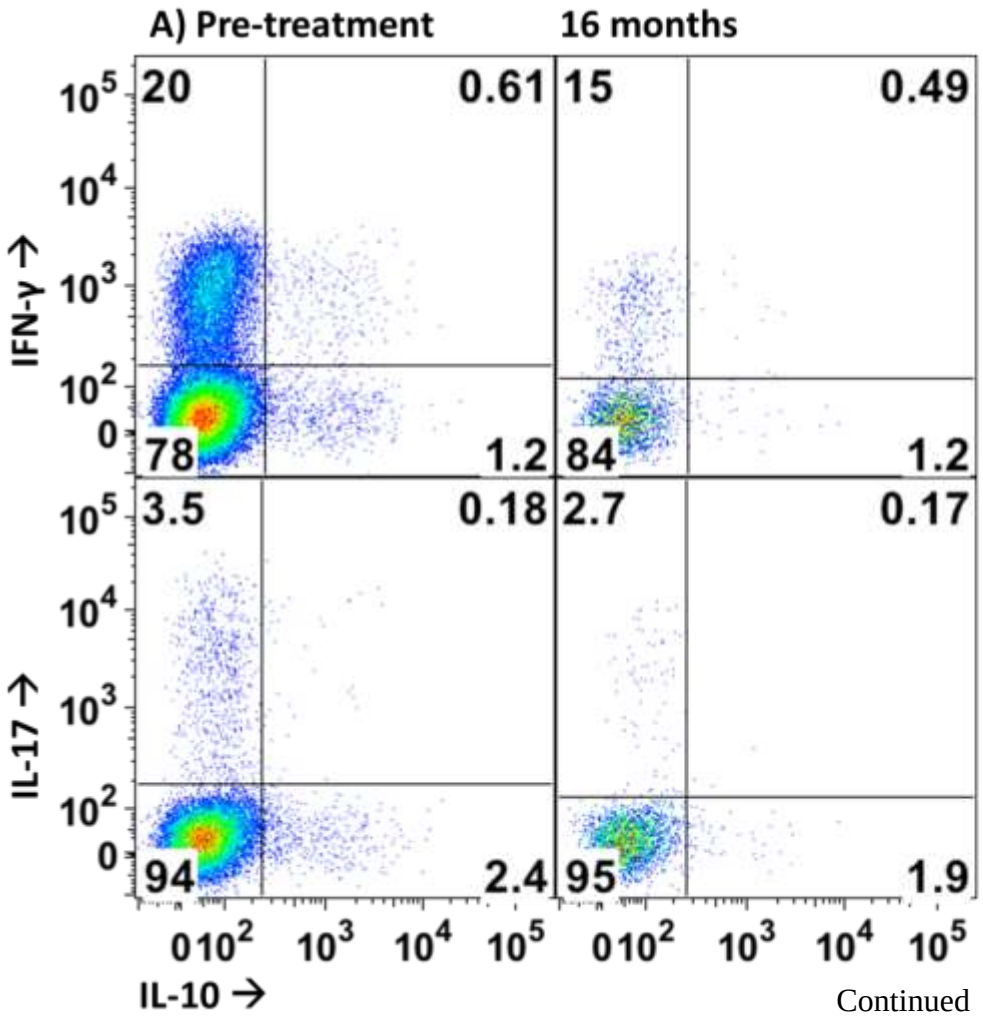

Figure VI.A: Cytokine profiles from IFN-β treated MS patients. Flow cytometry scatterplots from anti-CD-3/PMA/Ionomycin activated PBMC populations from 3 IFN-β (A, B, C) treated RRMS patients. Each page contains a single patient's data. Top to bottom, IFN-γ or IL-17 on Y-axis. IL-10 on all X-axes. Left to right, pre-treatment to post treatment. Gated as in Fig V. Axes are logarithmic.

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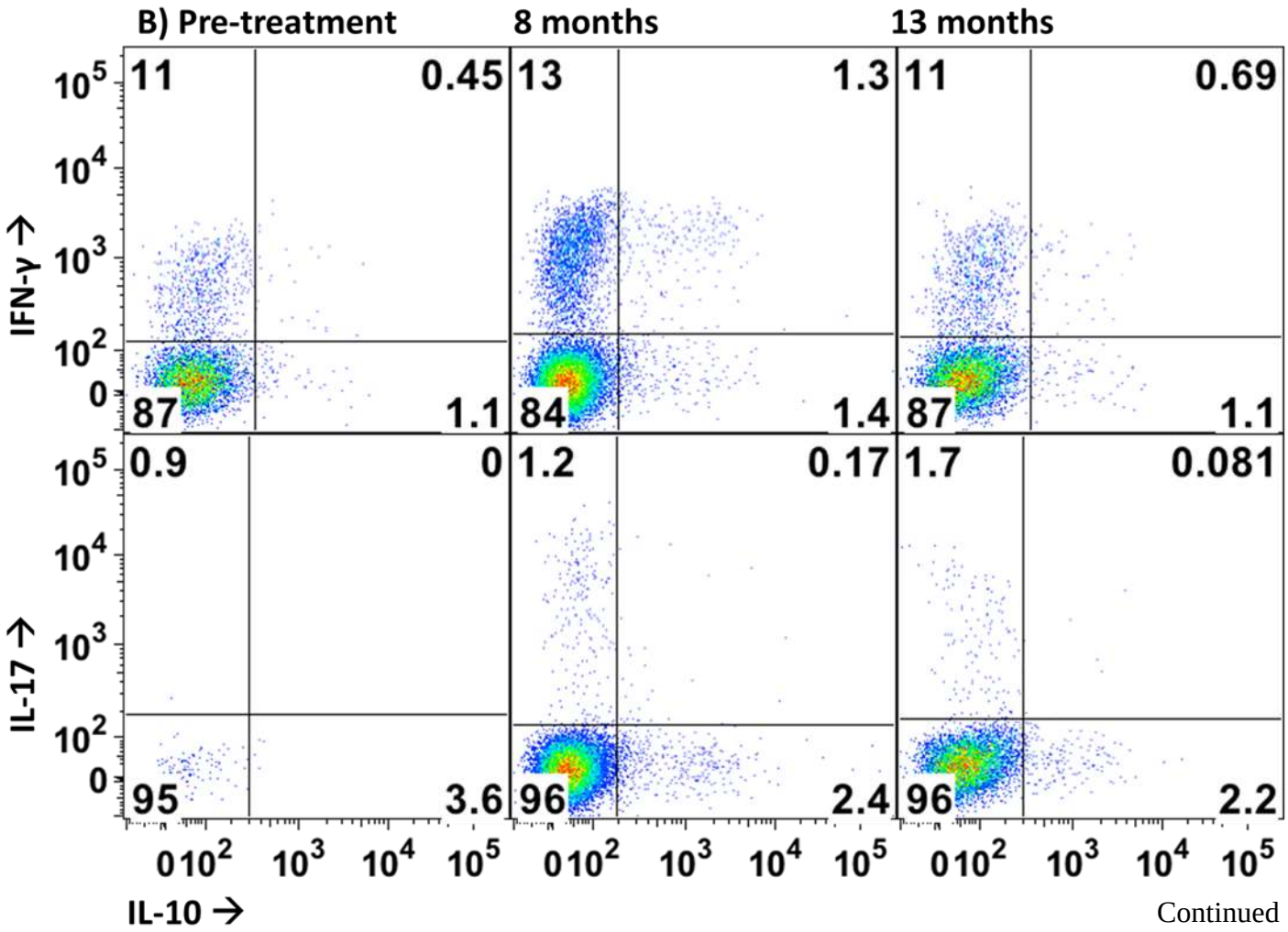

Figure VI.B: Cytokine profiles from IFN-β treated MS patients. Flow cytometry scatterplots from anti-CD-3/PMA/Ionomycin activated PBMC populations from 3 IFN-β (A, B, C) treated RRMS patients. Each page contains a single patient's data. Top to bottom, IFN-γ or IL-17 on Y-axis. IL-10 on all X-axes. Left to right, pre-treatment to post treatment. Gated as in Fig. V. Axes are logarithmic.

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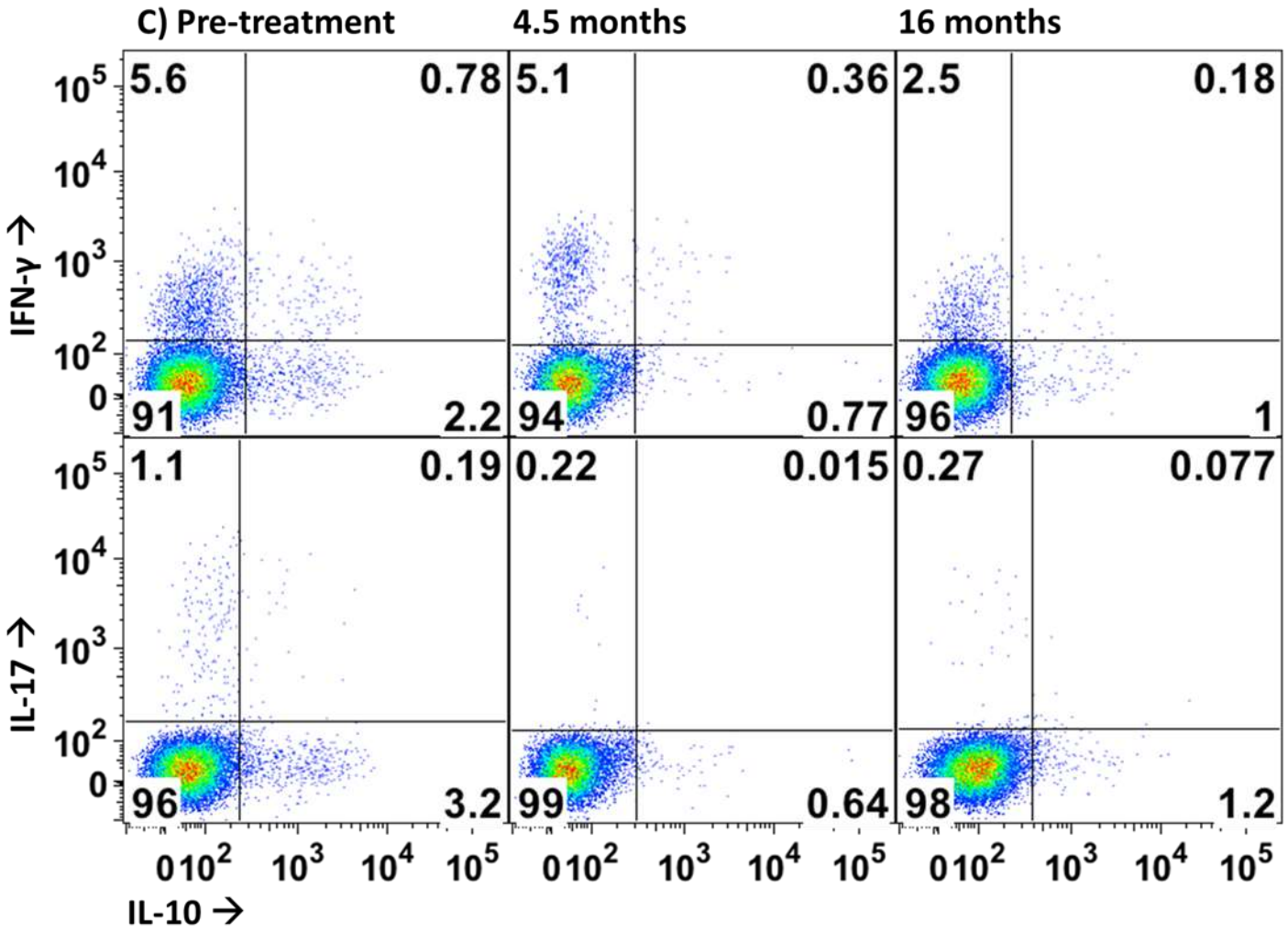

Figure VI.C: Cytokine profiles from IFN-β treated MS patients. Flow cytometry scatterplots from anti-CD-3/PMA/Ionomycin activated PBMC populations from 3 IFN-β (A, B, C) treated RRMS patients. Each page contains a single patient's data. Top to bottom, IFN-γ or IL-17 on Y-axis. IL-10 on all X-axes. Left to right, pre-treatment to post treatment. Gated as in Fig V. Axes are logarithmic.

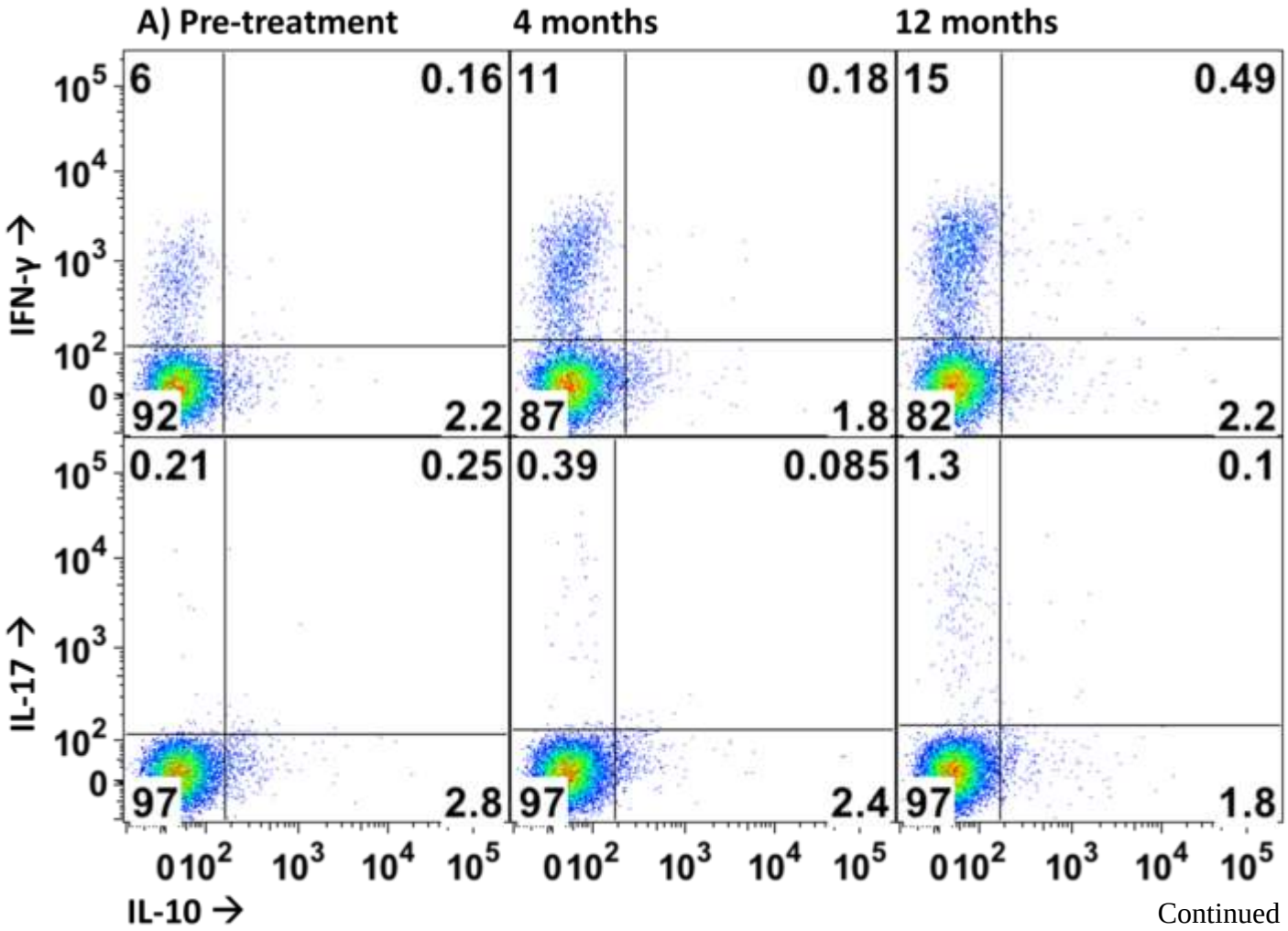

Figure VII.A: Cytokine profiles from copaxone treated MS patients. Flow cytometry scatterplots from anti-CD-3/PMA/Ionomycin activated PBMC populations from 2 copaxone (A, B) treated PPMS patients. Each page contains a single patient's data. Top to bottom, IFN-γ or IL-17 on Y-axis. IL-10 on all X-axes. Left to right, pre-treatment to post treatment. Gated as in Fig V. Axes are logarithmic.

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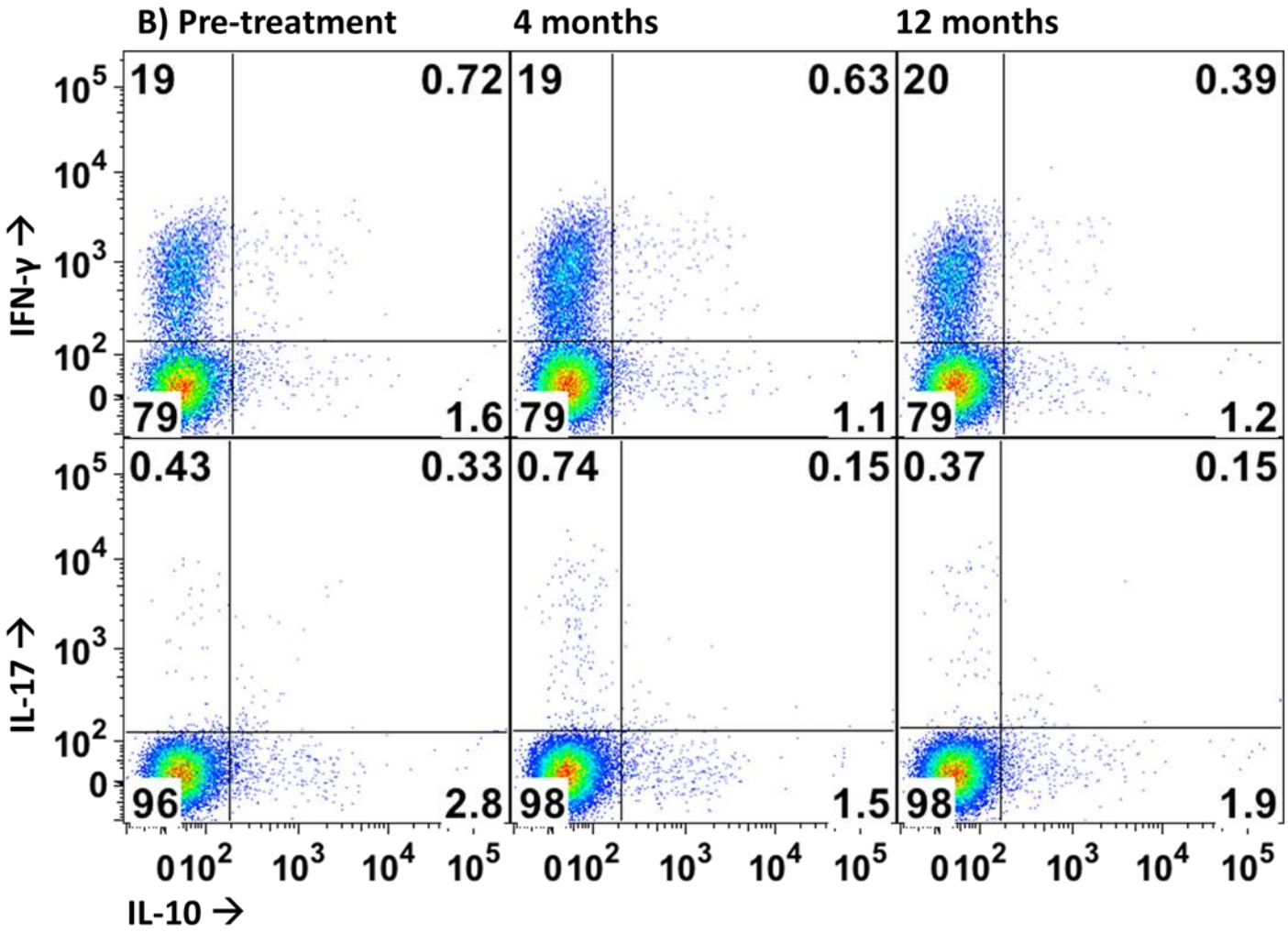
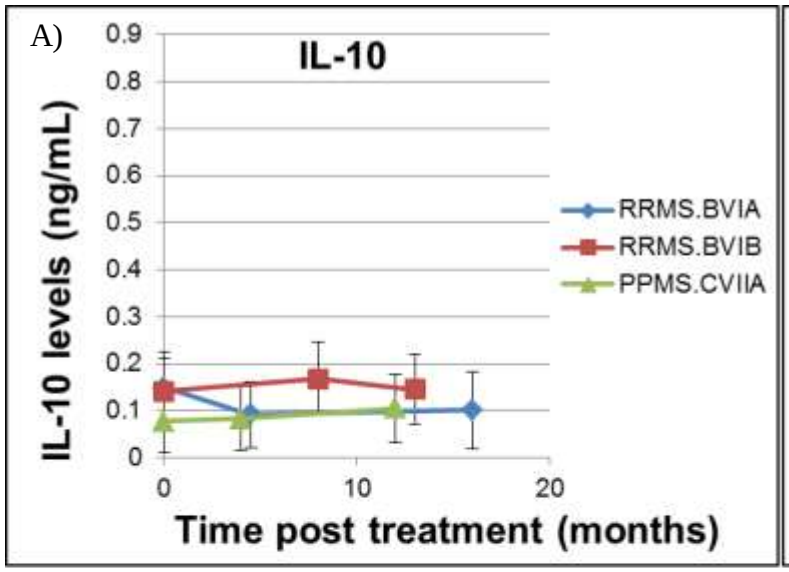
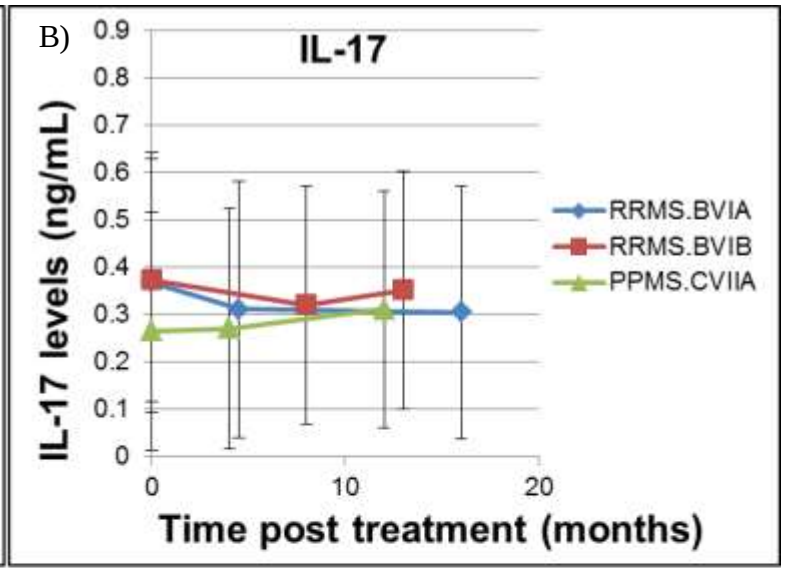


Figure VII.B: Cytokine profiles from copaxone treated MS patients. Flow cytometry scatterplots from anti-CD-3/PMA/Ionomycin activated PBMC populations from 2 copaxone (A, B) treated PPMS patients. Each page contains a single patient's data. Top to bottom, IFN-γ or IL-17 on Y-axis. IL-10 on all X-axes. Left to right, pre-treatment to post treatment. Gated as in Fig V. Axes are logarithmic.





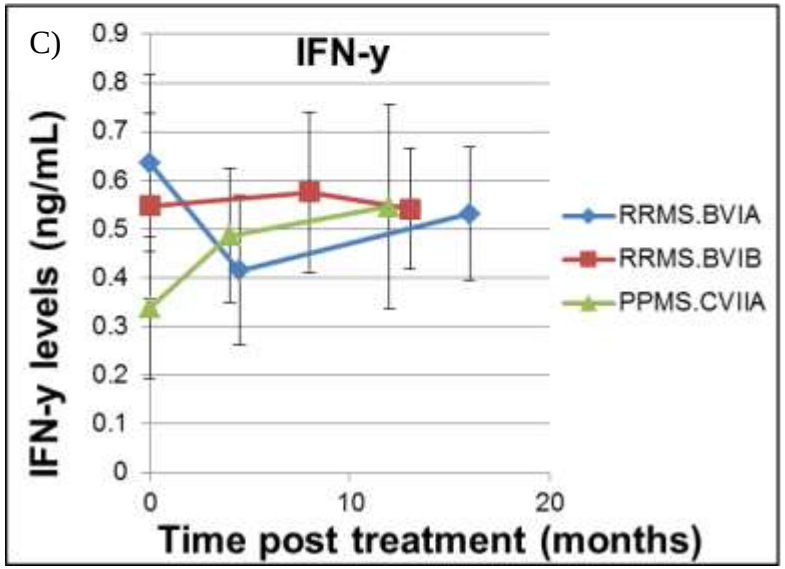


Figure VIII: ELISA data for copaxone and IFN-β treated cohorts. IL-10 (A), IL-17 (B), and IFN-γ (C). Presented as ng/mL versus time in months. Data presented is for the patients corresponding to flow panels VIA (RRMS.BVIA), VIB (RRMS.BVIB), and VIIA (PPMS.CVIIA). All samples performed in duplicate. Error presented as standard deviation. N=1. Note: VIB slightly diluted with PBS (within error).

Chapter 4: Discussion

Our data, thus far, does not confirm our hypothesis. While we did observe IFN-γ+/IL-10+ double positive cells, we did not see the expected increase in population over the course of IFN-β therapy. We have observed, primarily, a general decrease in cytokine positive cells over IFN-β therapy, which is one effect of that cytokine. In contrast, cytokine positive cells remain constant or even increased in the copaxone patient population, as seen with IFN-γ+ cells in Fig. VIIA. However, a difficulty pertinent to human studies is the issue of variation: day-to-day within a widely varying environment for each individual and between individuals (note a dramatic difference like that between the 20% IFN-γ+ cells for the patient in Fig. VIIB compared to the level for the others). Another potential flaw within our studies is the fact that only RRMS patients were used for the IFN-β studies and only PPMS for the copaxone studies. A more diverse patient population and a larger cohort may aid in identifying a role for IFN-γ+/IL-10+ cells in a specific patient subset. As illustrated by the literature earlier presented on IL-10 serum levels, there have been contradictory reports on serum IL-10[23,26]. This may reflect different populations. In the EAE model of MS, it has been shown that only Th1 cell driven EAE is responsive to IFN-β, while Th17 cell driven EAE is enhanced by IFN-β[28]. While it has not been shown that there a corresponding patient populations in MS, the

general principle of differing patient populations with different responses to IFN-β holds true. A larger cohort increases the chance of sampling the relevant populations.

Another direction our studies are taking is examining Tbet levels in IFN-γ+/IL-10+ cells. Tbet is the characteristic Th1 cell transcription factor and has been correlated with encephalogenicity in EAE[39]. By studying Tbet, we can further understand whether encephalogenic potential changes with IFN-β treatment in our target population. Currently, studies are ongoing to characterize Tbet levels in these cells, which may identify a $Tbet^{lo}$, IL-10+, IFN-γ cell subset, for example, that correlates with therapeutic efficacy. Future studies could also incorporate CFSE staining, a technique which measures dilution of the CFSE dye with each division of a cell, allowing further subdivision of populations. This would allow us to study whether the IFN-γ/IL-10+ population is overrepresented amongst the proliferative cells, as Huss *et al* demonstrated that, in the EAE model, this population is proliferative[34]. A role for IL-10+ Th1 cells in MS remains critical to elucidate because of the potential for therapeutic developments and biomarkers. Through further studies with an expanded cohort, we hope to identify a role for this population in amelioration of disease.

The importance of these studies lies in the potential to restore self-regulation by enhancing this population of IFN-γ+/IL-10+ Th1 cells. It has been demonstrated in mice that this population has therapeutic benefit and appears to be induced by chronic reactivation in a CNS-like environment[34]. Thus, this appears likely to be a major self-

regulatory mechanism. The fact that chronic infections exhibit IL-10+ effector T cell populations suggests that T cells default into this pathway in a chronic immune response[32,36]. In MS, such regulation must break down. Th1 cells are likely leading contributors to disease in MS, and by restoring their self-regulation, immunological equilibrium could be restored in patients. It is highly likely that only a subset of patients would exhibit such an effect, as over 30% of MS patients do not benefit from IFN-β therapy[28]. But by identifying this population, our studies could spur research in this direction and provide a basis for the use of IFN-γ+/IL-10+ cells as a biomarker for therapeutic efficacy. Such information would allow targeted use of appropriate therapies to specific patient populations. Our study also is furthering the study of IL-10+ effector T cells and promoting a better understanding of their role in human biology. Our techniques can be used to study other disease states in MS and other treatments, while it can also be extrapolated to other diseases. Through this study, we have elucidated a means to study human IFN-γ+/IL-10+ Th1 cells in disease.

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